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**ASSESSMENT OF DNA DAMAGE, OXIDATIVE STRESS
AND INFLAMMATION IN CHRONIC KIDNEY DISEASE
PATIENTS – AND A CLINICAL STUDY OF A DIETARY
SUPPLEMENT**

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ABSTRACT

Decreased kidney function is associated with higher levels of oxidative stress, inflammation and malnutrition. Chronic kidney disease (CKD) patients have a higher risk to develop cardiovascular disease, atherosclerosis and cancer compared to the general population. Cardiovascular disease is the major cause of death in CKD patients. Many CKD patients also report oral health problems including dry mouth symptoms, inflammation in the oral cavity and changes in the salivary constitution. These alterations can increase the systemic inflammation. CKD patients are often also deficient in several vitamins, due both an impaired kidney function, depletion during dialysis and decreased nutritional intake. In this thesis, including two clinical studies on CKD patients, levels of oxidative stress, inflammation, saliva production and blood markers were investigated. Oxidative stress was measured by analysis of oxidative DNA damage in salivary glands using the comet assay.

Paper I: The objective was to assess the levels of DNA damage in salivary gland biopsies, saliva production and inflammation in 79 CKD patients and compare the levels to controls. The relationships between the study parameters were investigated and the results for predialysis and dialysis patients were compared. The results showed that the dialysis patients had lower levels of DNA breaks and that predialysis patients had higher levels of DNA breaks compared to their controls. The saliva production was found to be lower in the dialysis patients compared to the control group as well as the predialysis group. The inflammation levels were found to be higher in CKD patients compared to the controls. Previous studies have shown raised levels of DNA damage in peripheral blood mononuclear cells from CKD patients. The results from this study suggest that the DNA in peripheral tissue in dialysis patients is affected differently.

Paper II: The objective was to investigate the effects of oral supplementation with sea buckthorn oil (SBO) on oxidative stress, saliva production and inflammation in hemodialysis patients. Sea buckthorn is rich in polyunsaturated fatty acids, vitamins and other phytochemicals. Positive health effects by SBO on dry eye symptoms, platelet aggregation and skin diseases have been reported. The 45 hemodialysis patients completed the 2 x 8 weeks placebo-controlled crossover study and the results did not show any effects on DNA damage, inflammation or saliva production. However, the levels of phosphate and sodium increased and iron levels decreased after SBO supplementation. The results from this study did not show any positive health effects of SBO supplementation on DNA damage, saliva production or inflammation.

In conclusion; oxidative stress and inflammation are important risk factors that contribute to disease progression and mortality in CKD patients. The interrelations between these events are complex and factors including dialysis treatment, medication, diet and oral health are of importance. In our study we found that despite elevated systemic inflammation, the levels of DNA damage in salivary glands in dialysis patients were lower compared to controls. The results suggest the involvement of DNA repair and antioxidative mechanisms in this tissue. Supplementation with SBO did not show any reduction on DNA damage or inflammation in dialysis patients, concluding that SBO supplementation did not have any beneficial health effects in our study group.

LIST OF PUBLICATIONS

- I. **DNA damage in salivary gland tissue in patients with chronic kidney disease, measured by the comet assay.**

Ersson C., Thorman R., **Rodhe Y.**, Möller L. and Hylander B.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011; 112:209-215

- II. **The effect of sea buckthorn supplement on oral health, inflammation and DNA damage in hemodialysis patients: a double-blinded, randomised crossover study.**

Rodhe Y., Bergström T., Thorman R., Möller L. and Hylander B.

Submitted

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LIST OF ABBREVIATIONS

A	Adenine
ALS	Alkali-labile sites
AP sites	Apurinic and apyrimidinic sites
ATP	Adenosine-5'-triphosphate
BER	Base excision repair
CKD	Chronic kidney disease
CRP	C-reactive protein
CVD	Cardiovascular disease
C	Cytosine
DNA	Deoxyribonucleic acid
ESRD	End stage renal disease
EtBr	Ethidium bromide
FPG	Formamido pyrimidine DNA glycosylase
G	Guanine
GFR	Glomerular filtration rate
hs-CRP	High sensitive C-reactive protein
IL	Interleukin
LPC	Leukocyte particle concentration
NER	Nucleotide excision repair
Ogg1	8-oxoguanine DNA glycosylase
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEW	Protein-energy wasting
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SBO	Sea buckthorn oil
SOD	Superoxide dismutase
SSB	Single strand break
T	Thymine
TNF	Tumour necrosis factor

1 INTRODUCTION

1.1 OXIDATIVE STRESS

Oxygen is essential for the energy production in humans. In the mitochondria, electrons are transferred from reducing agents to O_2 , forming H_2O as the final end product. In this electron transport chain, energy is conserved to synthesise adenosine-5'-triphosphate (ATP), the energy source of the cell. This reaction is tightly controlled by enzymes, however, electrons occasionally leak from the mitochondria, creating reactive oxygen species (ROS). Aerobic living cells are thus constantly exposed to ROS during the metabolism. ROS have the potential to oxidise and damage cellular macromolecules like DNA, proteins and lipids. Aerobic organisms have therefore evolved an antioxidant defence to counteract the oxidative burden. Oxidative stress is a term to describe an imbalance between the oxidants and the antioxidative defence in a biological system [1]. Even though ROS are potentially hazardous for the cell, they are also essential for the cell. Many signalling pathways are dependent on ROS and they play important roles in several redox-regulated processes. Pathogen defence is also a source of ROS and crucial for the cell; white blood cells produce ROS in response to stimulation by foreign agents. Beyond the endogenous production of ROS, formation can also be induced by extra cellular sources such as radiation and exposure to toxic compounds, drug metabolites and air pollutants including tobacco smoke, nanoparticles, diesel exhaust and ozone.

1.1.1 Formation of reactive species

Molecular oxygen (O_2) is transported from the air via the lung to tissue and cells by hemoglobin in the blood. The binding of O_2 to the protein is mediated by a heme group, containing Fe^{2+} that is consequently oxidised to Fe^{3+} . In the cell, O_2 is the final electron acceptor in the production of energy. Electron donors (e.g. NADH, $FADH_2$) derived from metabolic pathways, are used for single step reductions of O_2 (Figure 1), coupled to proton pumping and ATP synthesis in the mitochondria. In this electron transport, leakage of electrons occasionally occurs and as a consequence, ROS are generated. The mitochondria is considered as the major source of ROS in the cell.

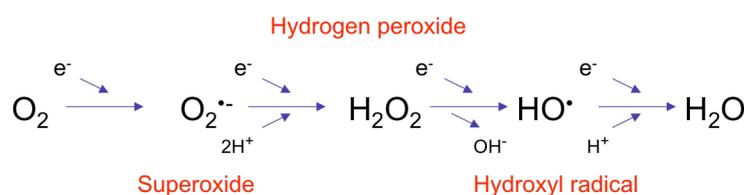
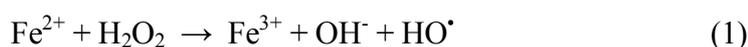


Figure 1. The stepwise reduction of molecular oxygen to water.

ROS include both radical and non-radical species. A free radical can be classified as any specie with one or more unpaired electrons in its atomic or molecular orbital. Most of the free radicals are highly reactive, with some exceptions. While molecular oxygen can be classified as a free radical containing two unpaired electrons (a diradical) with parallel spins, the reactivity is limited due to spin restrictions. The superoxide anion, $O_2^{\bullet-}$, is a free radical with one unpaired electron and it does generally not oxidise biomolecules in aqueous solutions. However, it can react with other radicals and oxidise Fe-S clusters, thus inactivating enzymes [2]. Dismutation of $O_2^{\bullet-}$ generates hydrogen peroxide, H_2O_2 , a relatively weak redox reactive agent. However, H_2O_2 can be cytotoxic, inactivate enzymes and it is also a potential source of the extremely reactive hydroxyl radical, HO^{\bullet} , by reacting with transition metals in the Fenton reaction (1).



HO^{\bullet} is extremely reactive with a rate constant of $1 \times 10^{10} M^{-1}s^{-1}$ for the reaction with guanine, and it can oxidise any cellular macromolecule close to its formation site, inducing radical chain reactions [1]. The rate of reaction is often only limited to the rate of diffusion of the reactants.

Other central ROS are ozone (O_3), singlet oxygen (1O_2), hypochlorous acid (HOCl) and peroxy (R-OO $^{\bullet}$). Reactive nitrogen species (RNS), including nitric oxide (NO $^{\bullet}$), nitrogen dioxide (NO $_2^{\bullet}$) and peroxynitrite (ONOO $^-$) are also important sources of oxidative stress.

1.2 OXIDATIVE DAMAGE

Oxidative stress, caused by a weak antioxidant system and/or an increased ROS production can cause damage to all the biomolecules in the cell. Oxidative damage to

the DNA, proteins and lipids can cause mutations and dysfunctions of the molecules. Several diseases, including cancer, atherosclerosis, cardiovascular diseases, chronic kidney disease and diabetes have been associated with oxidative stress.

1.2.1 DNA damage and repair

The DNA is responsible for storing genetic information in human cells. It is also subject to damage and instability, processes involved in both ageing and disease development. The integrity of the genome can be compromised by oxidising agents, alkylating agents and ionising radiation that induce modifications to the DNA. The major forms of DNA damage include DNA breaks, cross-links, base lesions, bulky adducts and modifications of the backbone. Cellular responses to DNA damage are DNA repair, apoptosis, cell cycle arrest or changes in the transcription of genes. Failure in the cellular response may lead to mutations and cancer.

Single strand breaks occur constantly in the cell and one major cause is oxidative attack. The break can either be direct, mediated through metabolites or as a consequence of enzymatic repair. All four bases are susceptible for oxidative damage. Guanine has the lowest redox potential and it is therefore the most readily oxidised base. The most studied oxidation product of guanine in DNA is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) which is formed by oxidation at the 8-position of 2'-deoxyguanosine (dG) (Figure 2) [3]. This oxidised form can assume *syn* conformation and base pair with adenine, causing a transversion of GC \rightarrow TA. 8-oxodG is a common biomarker of oxidative stress and due to its pro-mutagenicity it is also a potential biomarker of carcinogenesis [4].

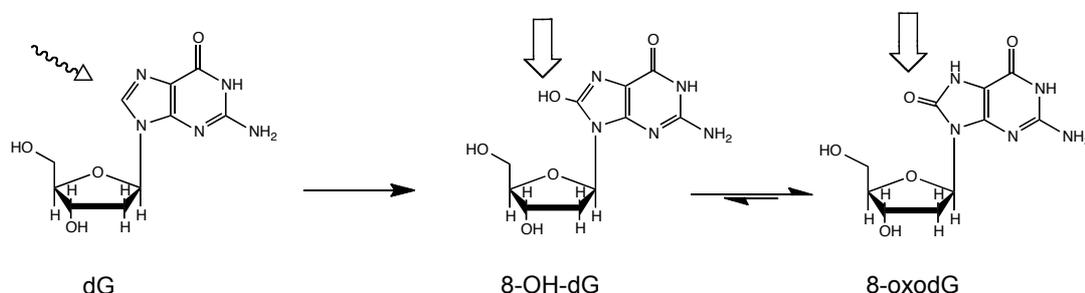


Figure 2. Structures of dG and the oxidation product 8-oxodG.

The cell has evolved a DNA repair system consisting of several groups of enzymes to protect the cell from genetic damage. There are excision repair pathways that remove the damage prior replication, mismatch repair systems that correct wrongly incorporated bases after replication and also homologous or non-homologous recombination that repair double strand breaks. In base excision repair (BER), the damaged base is recognised by a glycosylase that via hydrolysis of the N-glycosyl bond, removes the base, creating an apurinic or apyrimidinic (AP) site. After AP endonuclease or AP lyase activity, DNA polymerase can insert the appropriate nucleotide and the repair pathway is completed by ligase activity and sealing of the DNA. In humans, 8-oxoguanine DNA glycosylase (Ogg1) is an enzyme with both glycosylase and lyase activity, responsible for the removal of oxidised bases, including 8-oxoG [5]. Formamido pyrimidine DNA glycosylase (FPG) is a corresponding enzyme in *Escherichia coli* and is often used to quantify oxidised DNA lesions in DNA analysis, including in the comet assay [6] that was used in the studies of this thesis.

In nucleotide excision repair (NER), bulky adducts e.g. formed by UV light or chemicals are removed. The enzyme system responsible for this excision nuclease activity starts with the recognition of the damage, followed by the hydrolysis of the phosphodiester bonds on both sides of the adduct and the release of an oligomer containing 24-32 nucleotides [7]. The gap is then filled and the nicks are ligated by DNA polymerase and DNA ligase.

1.3 ANTIOXIDATIVE DEFENCE

To counteract the oxidative load and avoid oxidative damage, human cells have evolved an antioxidative defence. This defence includes mechanisms to prevent the formation of ROS, neutralise ROS after formation or mechanisms to repair the oxidative damage. It consists of enzymes, proteins and low molecular weight molecules such as glutathione and dietary antioxidants.

1.3.1 Antioxidant enzymes and proteins

Superoxide dismutase (SOD), catalase, glutathione peroxidase and peroxiredoxins are examples of antioxidant enzymes preventing damage by direct removal of ROS. SOD catalyses the dismutation of the superoxide anion to O₂ and H₂O₂. The human classes of this enzyme include CuZn-SOD (present in the cytosol, in the intermembrane space of the mitochondria as well as extracellular) and Mn-SOD (present in the mitochondria)

[8, 9]. H_2O_2 is eliminated in cells mainly by two types of mechanisms, dismutation and reduction, and three families of enzymes are known to be involved. The catalases catalyse the dismutation of H_2O_2 to H_2O and O_2 . Glutathione peroxidase is a family of enzymes reducing H_2O_2 to H_2O (or lipid peroxides to alcohols) by a selenocystein-based mechanism, and subsequently glutathione is oxidised. Glutathione-S-transferase is a non-seleno-dependent glutathione peroxidase reducing lipid peroxides but not H_2O_2 . The peroxiredoxin is a family of enzymes catalysing the same reactions through cystein-based activities. Since H_2O_2 is an important molecule for cell signalling, the elimination of H_2O_2 is well regulated.

Several proteins act as antioxidants by limiting the presence of free metal ions that otherwise could take part in Fenton reactions resulting in formation of the extremely reactive hydroxyl radical. Since both copper and iron are essential for many proteins in the cell, the management and transport of the ions are tightly controlled to avoid pro-oxidant effects. Transferrin, lactoferrin, ferritin, caeruloplasmin and albumin are examples of proteins that are sequestering and managing transport of iron and copper ions.

1.3.2 Dietary antioxidants

Dietary derived antioxidants include vitamins, carotenoids, flavonoids and other phytochemicals. Vitamins are small organic molecules required in human diet, due to a lack of capacity to synthesise them in sufficient amounts [10]. Vitamins often have a broad range of functions and many of them have antioxidative properties. Vitamin C, well known as ascorbic acid, is a water-soluble nutrient that has two ionisable OH-groups and at physiological pH the predominant form is the mono-anion ascorbate [1]. It is a cofactor for several enzymes needed for the proper biosynthesis of collagen and carnitine. While most animals and plants are able to synthesise ascorbate from glucose, humans lack this ability and need to ingest vitamin C through the diet. Lack of vitamin C can cause scurvy, a deficiency disease that leads to defect collagen [11]. Plasma levels of vitamin C are in the range of 50-60 μM for healthy individuals but the intracellular concentration can reach 1 mM in several cell types [12]. Ascorbate has a reducing ability and can act as a scavenger of ROS and RNS. The one-electron oxidation of ascorbate generates the ascorbyl radical that can be further oxidised to dehydroascorbate. The antioxidant effect of ascorbate is due to the replacement of damaging radicals by the less reactive ascorbyl radical. Ascorbate can also interact with

the radical species from α -tocopherol and glutathione, regenerating the antioxidant molecules [11]. Recommended daily intake of vitamin C for healthy adults in Sweden is 75 mg [13].

The carotenoids, including β -carotene, is a group of red and yellow coloured pigments that contain 40 carbon atoms. They can be found in a variety of fruit and vegetables and are important for humans as precursors of vitamin A. Vitamin A is a designation of a group of fat-soluble compounds that includes retinol, retinaldehyde, retinoic acid and retinyl esters. They all have vitamin A-activity and are essential for many biological processes in humans, including the immune system, vision, cell growth and cell differentiation [1]. Vitamin A is ingested through the diet as retinyl esters, from animal sources, or as provitamin A from plant sources [14]. The Swedish dietary recommendations for vitamin A compounds are 700 - 900 retinol equivalents (1 retinol equivalent = 1 μ g retinol = 12 μ g β -carotene) per day for healthy adults [13]. β -carotene is the most important provitamin A and must be oxidatively cleaved in the intestine to achieve vitamin A-activity [1]. Retinal, the formation product, can further be reversibly reduced to retinol or oxidised to retinoic acid [14]. The carotenoids can act as antioxidants by quenching singlet oxygen. This property depends on the length of the conjugated double bond chain. β -carotene can also scavenge peroxy radicals, forming an unstable β -carotene radical adduct that further can generate non-radical products [15]. However, if the oxidation products of β -carotene are not neutralised by other antioxidants, they can have pro-oxidative effects in the cell.

Eight fat-soluble tocopherols and tocotrienols derivatives have been found to have vitamin E activity. Their chemical structure includes a chromanol ring with one to three methyl groups and a sidechain that contains either three double bonds (tocotrienols) or a phytyl chain (tocopherols). Both types have four isomers, α , β , γ and δ . The α -tocopherol is considered to be the most bioavailable vitamin E in humans [16]. Recently more focus on the other vitamin E compounds has been raised. The vitamin E compounds are able to scavenge peroxy radicals, thereby inhibiting the free-radical chain reaction of lipid peroxidation. Tocopherols can also show pro-oxidant effects, in the way that the α -tocopherol radical can react with a polyunsaturated fatty acid, starting a lipid peroxidation chain reaction. Nevertheless, the rate constant is much lower than for the peroxy radical to react with the polyunsaturated fatty acid and the importance of this α -tocopherol-mediated peroxidation *in vivo* is questioned. Vitamin A can prevent such oxidation by recycling the α -tocopherol radical [17]. Tocopherols

can also protect cellular membranes against singlet oxygen by quenching [17]. The Swedish dietary recommendations for vitamin E compounds are 8-10 α -tocopherol equivalents (1 α -tocopherol equivalent = 1 mg RRR- α -tocopherol) per day for healthy adults [13].

1.4 ANALYSIS OF OXIDATIVE STRESS

ROS react fast and are short-lived *in vivo*. Due to this, measurements of the actual ROS levels are difficult and it is more feasible to assess oxidative stress by measuring the levels of oxidation products. The assessment of oxidative stress in this thesis is based on analysis of oxidative DNA damage, mainly 8-oxoG, using the comet assay. Other biomarkers of oxidative stress include malondialdehyde, isoprostanes and carbonylated proteins.

1.4.1 The comet assay

Single cell gel electrophoresis, commonly called the comet assay, is a method that can be used to assess DNA damage in eukaryotic cells. Cells from blood, tissue or cell cultures can be analysed. The alkaline comet assay, firstly described by Singh *et al* [18], is often modified to include the following general steps. Initially, a single cell suspension is required and when analysing tissue, single cell suspensions can be obtained either by homogenisation or by enzymatic degradation. The cells are then lysed during which the cell membrane is destroyed and most of the proteins are disrupted. Treatment with lesion-specific enzymes to enable detection of different DNA damages is a common modification of the comet assay [6]. In this thesis FPG was used to detect oxidative lesions, FPG-sensitive sites. This enzyme detects oxidised purines, mainly 8-oxoG, fapy-G and fapy-A, and exerts its glycosylase function by cutting the bond between the base and the sugar backbone [19]. This site is then transformed to a strand break by the lyase activity or during the alkali treatment. Alkali treatment is performed to unwind the DNA double helix and obtain single stranded DNA in order to detect single strand breaks (SSB). It also enables alkali-labile sites (ALS) to form SSB by hydrolysis. During the electrophoresis, the negatively charged DNA loops migrate under the electric field out of the nucleoid towards the anode, forming the tail of the comet. The migration is dependent on the level of damage.

Staining the DNA enables visualisation and assessment of the amount of DNA in the head and the tail of the comet, hence an estimation of the level of DNA damage

is feasible. Ethidium bromide (EtBr) has been used in this thesis and is a fluorescent dye that binds to DNA. Other dyes that are frequently used are Sybr® safe, Sybr® gold or DAPI (4',6-diamidino-2-phenylindole). Using a fluorescent microscope, the comets can be scored manually or automatically using software that provides information on % DNA in tail (preferably used) or other measure units (tail length or tail moment). In this thesis %-DNA in tail was measured using a software for computerised image analysis (Komet 4.0; Kinetic Imaging Ltd).

1.5 CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD) is a worldwide public health problem. In the US, more than 10 % of the population over 20 years, almost 20 million people, are estimated to have a decreased kidney function [20]. In the beginning of year 2010, over 570,000 patients in the US were being treated for End-Stage Renal Disease (ESRD), including hemodialysis treatment (370,274), peritoneal dialysis treatment (27,522) and kidney transplanted (172,553) patients [21]. In Sweden, 8,501 patients were included in uremic care at the end of year 2010. This number includes patients undergoing hemodialysis (2,920), peritoneal dialysis (841) and patients with kidney transplant (4,740) [22].

Both the kidney disease and the renal replacement therapy significantly affect the life of CKD patients. Dialysis treatment results in a major loss of both life-quality and economical income for the patient. Hemodialysis treatment involves treatment sessions for several hours at medical care centres, three times per week. Cardiovascular disease is the major cause of death in CKD patients and it is strongly associated with decreased kidney function [23]. Glomerulonephritis, diabetes nephropathy and polycystic kidney disease are common causes of kidney failure in Sweden [22].

1.5.1 Kidney function

The kidneys accomplish several functions to purify the blood, produce urine and maintain a homeostatic extracellular milieu. Kidney function is essential for the excretion of waste products such as creatinine and urea that are formed during the metabolism. In addition, many drugs and drug metabolites are excreted by the kidney. The kidney regulates the water and electrolyte balance by controlling the excretion and reabsorption of water, potassium, sodium, phosphate, calcium and other substances. Maintaining the balance in electrolytes and water is important for the regulation of blood pressure and body fluid volume. The acid-base status of the blood is also

controlled by urinary excretion of protons. Further, the kidney exerts an endocrine role, producing hormones including erythropoietin, renin, prostaglandins and vitamin D. The functional unit of the kidney is the nephrons and they are responsible for the filtration of the blood in the renal capillaries. Glomerulus is the filtration unit that produces urine and in the tubules the reabsorption and secretion of substances takes place. In a healthy individual, each kidney has between 400,000-800,000 nephrons, a number that decreases with age [24].

1.5.2 Kidney failure and disease

CKD is a progressive loss of nephrons and a decrease of renal function. This will lead to an augment of uremic symptoms including increased levels of urea and creatinine in the blood, disturbances in the electrolyte and water balance. Furthermore, the reduction of erythropoietin production can cause anaemia and the impaired vitamin D synthesis can cause hyperparathyroidism. The disease is preferably determined by measuring the glomerular filtration rate (GFR) and is often classified into different stages according to the National Kidney Foundation (Table 1) [25]. At stage 1, the kidney function is normal and in stage 2, the function is mildly reduced. These stages of disease might not be noted by the person and do not always cause uremic symptoms. However, in both stages signs of kidney disease such as proteinuria, haematuria, structural abnormalities of the kidney or genetic diagnosis can be found and diagnosis is of importance for anticipating and preventing disease progress and risk for cardiovascular disease. Patients at stage 3 have a moderately reduced kidney function with an increased need of dietary restrictions, medical care and monitoring of the disease. At stage 4, the kidney function is severely reduced and at stage 5, the disease is life-threatening and renal replacement therapy is necessary for survival.

CKD stage	GFR*	Description
1	>90	Normal kidney function, signs of kidney disease
2	60-89	Mildly reduced kidney function, signs of kidney disease
3	30-59	Moderately reduced kidney function
4	15-29	Severely reduced kidney function
5	<15, dialysis	Very severe kidney failure

Table 1. Classification of chronic kidney disease according to the US National Kidney Foundation. (* mL/min/1.73 m²)

ESRD is managed by protein-reduced diet, medication for hypertension and correction of electrolytes, renal replacement therapy including dialysis treatment or kidney transplantation. Transplantation is often desired, however, it is not suitable for all patients and due to shortage of donors, the waiting list for kidney transplantation can be a few years. Dialysis treatment is performed to replace an impaired kidney function. This is an artificial purification of the blood where waste products are removed and the balance between salts and fluids is regulated. In hemodialysis, the blood is pumped out of the patient's body and filtered using a dialyzer before it returns to the patient. The dialyzer is designed with a semi-permeable membrane allowing passage of substances and fluids between the blood and the dialysate. In peritoneal dialysis, the exchange of substances and fluids is performed using the patient's peritoneal lining as a filter inside the abdomen. A catheter is used to transfer the dialysis solution in and out of the peritoneal cavity [24].

1.5.3 Oxidative stress and inflammation in kidney disease

Kidney failure is associated with higher levels of oxidative stress, inflammation and malnutrition that contribute to the higher risk for cardiovascular diseases, atherosclerosis and cancer [26-28]. Elevated levels of oxidative stress are frequently reported in CKD patients [29-32]. This imbalance can occur as a consequence of both an increased production of ROS and insufficient antioxidant defence. Beyond the generation of ROS during normal cellular metabolism, potential sources of oxidative stress in CKD patients also include systemic inflammation, incidence of diabetes, dialysis treatment, reduced levels of dietary intake of antioxidants and the uremia itself. Inflammation, oxidative stress and malnutrition are closely related in renal failure and contribute to the increased risk for cardiovascular disease and mortality. Raised levels of inflammation markers such as specific cytokines and acute-phase reaction proteins are observed in CKD patients [29-31]. Commonly used inflammation markers in blood include C-reactive protein (CRP), interleukins, albumin, fibrinogen, amyloid A and tumour necrosis factor- α [33].

The dialysis treatment itself is also a source of oxidative stress and inflammation. The contact between the blood and the dialysis membrane (that can be more or less biocompatible depending on material) will cause alterations in the constitution of blood cells during hemodialysis. The alternate pathway of the complement system can be activated, demonstrated by analysis of the activation products C3a and C5a [34]. Memoli *et al* showed that PBMCs harvested after

hemodialysis with cuprophane membrane produced higher levels of IL-12 compared to both controls and patients dialysed with a polymethylmethacrylate membran [35]. In the process of neutrophil activation, the superoxide anion is formed by the NADPH oxidase complex throughout respiratory burst as a part of pathogen defence. Furthermore, degranulation of neutrophils also triggers ROS production. Contaminated dialysate containing endotoxins and bacterial cell wall fragments, can also stimulate the activation of monocytes and production of cytokines such as IL-1 and tumour necrosis factors with pro-inflammatory effects [36].

The prevalence of anaemia, a decrease in red blood cells, is higher in CKD patients and can be caused by reduced production of erythropoietin (a hormone important for red blood cell production) by the kidney, lack of folate or vitamin B₁₂, or iron deficiency. Impaired erythropoiesis can be improved by erythropoiesis-stimulating-agents and supplementation with oral or intravenous iron. Iron supplementation has also been discussed as a potential source of oxidative stress since free iron, not bound to iron-binding proteins such as transferrin, is a possible participant in Fenton reactions that generate hydroxyl radicals. Lipid peroxidation has been shown to be increased shortly after intravenous iron infusion [37, 38]. However, the significance of these pro-oxidative effects is uncertain and the benefits of iron supplementation on iron repletion and anaemia are central [39]. Intravenously iron supplementation in appropriate dose and preparation is widely used and recommended [40].

1.5.4 Malnutrition

Malnutrition and protein-energy wasting (PEW) are considered to be additional risk factors for the high mortality in CKD patients [41] [42]. PEW is used to describe a state where the storage of protein and body fat is depleted [43]. This will lead to loss of body weight and muscle mass. The prevalence of PEW among CKD patients is higher as a consequence of several factors including inadequate nutritional intake, malnutrition and uremic effects such as uremic toxins, increased inflammation and hormonal disturbances. Hypoalbuminemia is commonly used as a marker for malnutrition and PEW [43]. It is also considered as a negative marker for acute phase reactions, thus illustrating the close interrelation between inflammation and malnutrition in CKD patients.

Dietary restrictions for CKD patients may be required to diminish the complications of kidney failure and dialysis treatment. The purpose is to decrease uremic symptoms and to keep the patient at a healthy weight by individual restrictions and dietary advice. Reduced intake of proteins, potassium, sodium, phosphorus, calcium and excess fluid are often recommended. Alterations in the diet, in some cases with a reduction of intake of fresh fruits and vegetables to evade hyperkalemia and limit levels of phosphorus and calcium, is a potential cause of antioxidant deficiency. In addition, dietary restrictions and decreased nutrient intake can enhance the state of PEW [43].

1.5.5 Oral health in CKD

Both kidney failure and dialysis treatment have impact on oral health. It is also known that in the general population, oral health affects the general health. Inflammation and complaints in the oral cavity contribute to systemic inflammation and the development of atherosclerosis and cardiovascular disease [44]. Several studies report impaired oral health status in CKD patients. The complaints include xerostomia (mouth dryness), mucosal lesions, gingival enlargement, periodontitis, tooth loss and changes in the saliva [45-48]. Potential causes of oral health problems among CKD patients include a reduced oral care by the patients, medication, altered diet with increased levels of carbohydrates and enhanced susceptibility to infections due to dysfunction of the immune defence caused by the uremia and dialysis treatment [49]. The saliva is essential for maintaining a good oral health. By coating the oral cavity it protects the oral tissue from infections and breakdown. The production of the saliva is managed by three pairs of major salivary glands and hundreds of minor salivary glands. Measurements of unstimulated or stimulated saliva production can provide information regarding salivary gland function and oral health status.

1.6 DIETARY SUPPLEMENTS

Epidemiological studies show that a diet rich in fruit and vegetables is inversely correlated to cancer, cardiovascular diseases and mortality [50, 51]. The positive health effects are often attributed to the vitamins and antioxidants present in large amounts in fruit and vegetables. Therefore, there is a large interest in dietary supplements containing these substances. However, results from intervention studies on vitamin supplements are inconclusive in showing beneficial effects on health, several studies do not show any health effects and some even show harmful effects [52, 53]. The

inconsistent results highlight the complexity in the topic of dietary supplementation, suggesting that there are other important components of fruits and vegetables that also play significant roles in disease prevention. The studies also indicate that there might be certain groups that would benefit more from dietary supplement, potentially groups that are poorly nourished and have low levels of antioxidants. In the Linxian study, with participants of poor nutritional health, the mortality decreased after supplementation with α -tocopherol, β -carotene and selenium [54]. In the French SU.VI.MAX study it was found that supplementation with multivitamins and minerals lowered the incidence of cancer and mortality in men. The same effect was not seen among women and it was suggested to be due to the lower baseline levels of vitamin C and β -carotene in men compared to women [55]. It must also be noted that some groups are more susceptible for increased health risks, e.g. smokers [52, 53].

1.7 SEA BUCKTHORN OIL

Recently, the sea buckthorn berry has raised more interest in the Western world, both as a part of the diet and as a substrate in health products. Sea buckthorn has traditionally been used as a medicinal plant for several hundred years in China, Turkey and Russia. The sea buckthorn berry contains high levels of unsaturated fatty acids, vitamin C, vitamin E, carotenoids and phytochemicals including flavonoids and other phenolic compounds. It has been attributed antioxidative properties, anti-tumour effects, anti-inflammatory effects and also immune response regulatory effects [56-59]. Most studies are *in vitro* studies of different extracts of the berry. A few clinical studies on both whole berries and extracts have also been published. Reported effects include inhibition of induced platelet aggregation [60], reduction of CRP-levels in serum [61] and attenuated increased tear film osmolarity in dry eye symptoms [62].

The berry can be found on the consumer market, as an ingredient in jam, juices and other food products. Different extracts of the sea buckthorn berry are also used as dietary supplement and as ingredients in skin products. There are seven subspecies of sea buckthorn with different geographical origin; *Hippophae rhamnoides L.* is the most frequent species in Europe.

1.7.1 Fatty acids

Fatty acids, carboxylic acids with varying length of unbranched carbon chains, are important to the cell for the structure of membranes, storing energy and as precursors of

hormones. Classification of fatty acids is based both on the number of carbon atoms and the number and position of any double bond in the chain. The number of double bonds determine whether the fatty acid is saturated (no double bond), monounsaturated (one double bond) or polyunsaturated (more than one double bond). Saturated fatty acids have a straight arrangement in the carbon chain whereas a double bond causes a kink of the chain, allowing packed structures to be less compact. Certain polyunsaturated fatty acids (PUFAs) including α -linolenic acid and linoleic acid are considered as essential fatty acids since humans can not produce sufficient amounts by themselves. Omega-3 fatty acids, containing a double bond at the third carbon from the methyl group, include the key derivatives α -linolenic acid (18:3 ω -3), eicosapentaenoic acid (20:5 ω -3) and docosahexaenoic acid (22:6 ω -3). Omega-6 fatty acids include linoleic acid (18:2 ω 6), γ -linolenic acid (18:3 ω -6), arachidonic acid (20:4 ω -6) and docosapentaenoic acid (22:5 ω -6). The derivatives from the two families of omega-3 and omega-6 fatty acids have been shown to play important roles in the immune system and in inflammation. Omega-6 can induce inflammation, mediated by prostaglandin 2 that is generated from arachidonic acid via activity of cyclooxygenase (COX) enzymes. This enables TNF- α induced transmigration of neutrophils across endothelial cells. Prostaglandin 3, generated from omega-3 fatty acids, can act as antagonist in this pathway, thus acting in an anti-inflammatory mode [63].

The type of fatty acid in sea buckthorn varies between plant origins and also between the different parts of the berry; the seeds are rich in linoleic, α -linolenic, oleic (18:1 ω -9) and palmitic (16:0) acids. Whereas the fruit flesh is rich in more saturated fatty acids including palmitic acid and palmitoleic acid (16:1 ω -7) [64].

2 AIM

The overall aim of this thesis was to examine oxidative stress, inflammation and oral health among patients with chronic kidney disease. Further, to investigate the possibility to affect these factors with dietary supplementation of a natural extract of sea buckthorn oil.

Specific aims:

- Assess the DNA damage and oxidative DNA damage in salivary glands in CKD patients.
- Investigate salivary secretion rates, inflammation and blood parameters in CKD patients.
- Investigate correlations between DNA damage in salivary glands with salivary secretion rates, inflammation and blood parameters.
- Investigate possible effects of a dietary supplement of sea buckthorn oil on oxidative stress, inflammation and oral health.

3 METHODS

3.1 STUDY PARTICIPANTS AND METHODS

3.1.1 Participants and study design

In the observational study (Paper I) the patient group was CKD patients with renal disease stages 4-5 including predialysis patients (patients with a GFR <20 ml/min/1.73 m², not yet on dialysis) and dialysis patients. The patients were recruited from the Department of Renal Nutrition at Karolinska University Hospital and from the dialysis units at Löwenströmska Hospital, Kungsholmsdialysen and Sophiahemmet, Stockholm, Sweden. Healthy control patients, age- and sex-matched, were recruited from the public dental service at Solna municipality. Patients with active hepatitis, earlier detection of MRSA or participation in other studies were excluded. 79 patients were included in the study (25 women and 54 men, mean age 60 years). 10 patients were predialysis patients, 3 were peritoneal dialysis patients, and 66 were hemodialysis patients.

The study was conducted to assess levels of DNA damage in minor accessory salivary glands, salivary secretion rates, inflammation and uremic markers in saliva and blood. Interactions between the parameters and potential influence of sex, age, smoking and diabetes were also investigated.

In the intervention study (Paper II) the patient group was hemodialysis patients recruited from the Department of Renal Nutrition at Karolinska University Hospital. The inclusion criteria were age above 19 years, hemodialysis treatment for more than three months, stable medication and original teeth. Patients with dysphagia, active hepatitis, earlier detection of MRSA or participation in other studies were excluded. The study approach was to investigate the potential effect of a dietary supplement of commercially available sea buckthorn oil extract (supercritical CO₂-extraction of both fruit flesh and seeds), on DNA damage in minor accessory salivary glands, inflammation and blood parameters. The study design was a randomised and double-blinded crossover study with 2 x 8 weeks treatment periods. To avoid carry-over effects, a four week wash-out period between the treatment periods was performed. 72 hemodialysis patients were included in the study and randomised into two groups, one group (AB) receiving SBO in the first treatment period and the other group (BA)

receiving placebo (Figure 3). The intake was instructed to four capsules per day and the content and daily dose can be seen in Table 2.

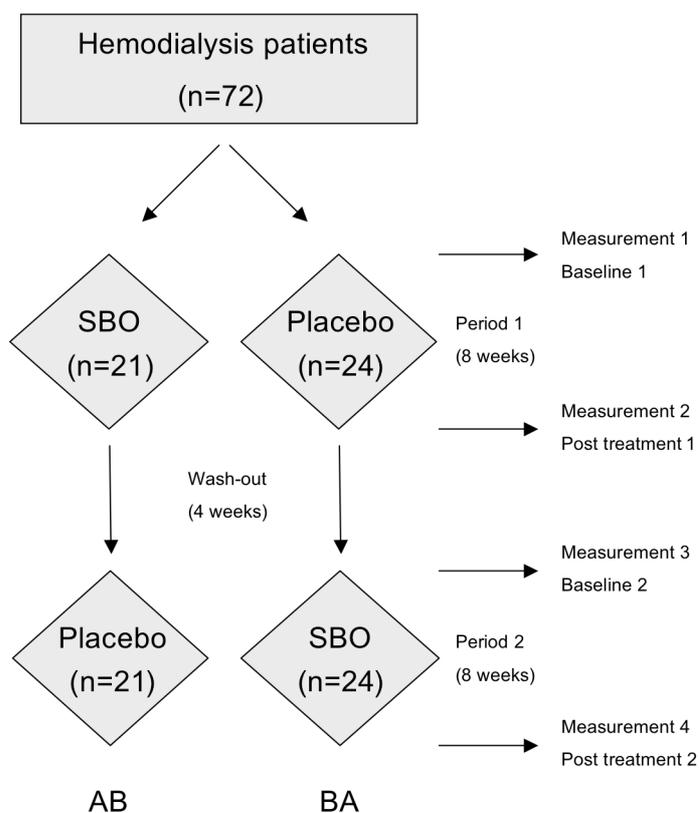


Figure 2. A crossover study design (2 x 8 weeks) with two sequence groups; AB receiving sea buckthorn oil and BA receiving placebo in the first treatment period (Paper I)

	1 SBO capsule (500 mg)	Daily dose (4 capsules, 2 g)
Oleic acid (C18:1 ω9)	124 mg (24.8%)	496 mg
Palmitoleic acid (C16:1 ω7)	97 mg (19.5%)	388 mg
Linoleic acid (C18:2 ω6)	92 mg (18.4%)	368 mg
α-linolenic acid (C18:3 ω3)	63 mg (12.6%)	252 mg
Vitamin E	931 μg	3.72 mg
Vitamin A	88 μg	352 μg

Table 2. Daily dose and capsule content according to the producer of the SBO capsule.

3.1.2 Ethical permissions

Both studies were approved by the regional Ethics Committee for Human Research at Karolinska Institutet and conducted in accordance with the Helsinki Declaration (2008).

3.1.3 Blood samples

Blood samples were collected venously and in Paper I, the levels of high sensitive C-reactive protein, albumin, urea and creatinine were measured in serum, leukocyte particle concentration (LPC) and hemoglobin in blood and haptoglobin in plasma. The proteins were quantified with near infrared particle immunoassay (Beckman Coulter, Brea, CA).

In Paper II, venous blood samples were collected before and after each treatment period. The following markers were measured in plasma (p) or blood (b): p-albumin, p-antitrypsin, p-calcium, p-calcium x phosphate product, p-carbon dioxide (CO₂), p-creatinine, b-erythrocytes, p-glucose, p-haptoglobin, b-haemoglobin, p-high sensitive C-reactive protein (hs-CRP), p-immunoglobulin A (IgA), p-immunoglobulin G (IgG), p-immunoglobulin M (IgM), p-iron, p-iron saturation, b-leukocytes, p-orosomuroid, p-phosphate, p-potassium, p-sodium, b-thrombocytes, p-transferrin and p-urea.

3.1.4 Salivary secretion rates

Measurement of the saliva production was made according to standardised methods. The patients were asked to lean forward and hold a funnel and a test tube to collect the produced saliva. For measurement of produced saliva at rest, the patients were asked to achieve a passive flow of saliva without masticatory movements for 15 min. For assessing the saliva produced under stimulation, the patients were asked to chew paraffin capsules and collect the produced saliva during 5 min. The level of salivary secretion rate was expressed in ml/min.

3.1.5 Gland collection

The salivary glands were collected from CKD patients and control persons by incision with scalpel (Number 15, Braun, Tuttlingen, Germany) under local anesthesia (Citanest Dental-octapressin 30%, Dentsply, Stockholm, Sweden). The region for the incision

was on the inside of the lower lip, close to an accessory gland, and the region was the same for each person. Immediately after the gland removal, the tissue was kept in physiological saline solution and frozen at -70°C prior DNA analyses. The incision was closed with an Ethicon suture 5.0 (Johnson & Johnson International, New Brunswick, NJ, USA) when needed.

3.1.6 Comet assay

After thawing, the salivary glands were homogenised in phosphate buffer saline (PBS) using a Dounce B Pestle. $27\ \mu\text{L}$ of the cell suspension was added to $210\ \mu\text{L}$ 37°C 0.75% low melting point agarose and the mixture was spread out to thin layers on microscope slides. Each gland was prepared in duplicate for analysing both strand breaks and alkali-labile sites (SB and ALS) and FPG-sensitive sites. After solidifying of the gels on a cold plate, the slides were put in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10 and 1 % Triton X-100) for 60 min on ice and in dark. After washing the slides 3 x 5 min in enzyme buffer (0.1 M KCl, 40 mM HEPES, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8), $30\ \mu\text{L}$ FPG enzyme in enzyme buffer (FPG-sites) or only enzyme buffer (SB and ALS) was added to the slides and incubated in a humidity chamber at 37°C for 30 min. The slides were then put in an alkali solution (0.3 M NaOH, 1 mM EDTA) for 40 min prior to electrophoresis. The electrophoresis was performed in alkali solution in a black electrophoresis tank with a cooling system of ice-cold water circulating under the platform of the tank. The duration of the electrophoresis was 30 min and the applied electric field was 1.15 V/cm. After the electrophoresis, the slides were put for 2 x 5 min in TRIS (0.4 M, pH 7.4) for neutralisation and 5 min in H_2O . The slides were allowed to dry over night and then put in methanol for 5 min to dehydrate prior to DNA staining.

The slides were stained with ethidium bromide solution (1 $\mu\text{g}/\text{mL}$) for 5 min and analysed using a fluorescence microscope (Olympus BH-2 with a 20x apochromatic objective).

Scoring was performed using the software Komet 4.0 (Kinetic Imaging Ltd) and the % DNA in tail was measured for the assessment the DNA damage. By subtracting the % DNA in tail of the non-FPG-treated slides from the FPG-treated slides, the level of FPG-sensitive sites was assessed. For each gland and sample, 105 cells were scored in total, 35 cells on each of 3 fields on the microscope slide.

To verify the stability of the comet assay, one aliquot of PBMCs from one single donor was used for each day of comet assay analysis. The isolation of PBMCs from whole blood was performed with a Histopaque-1077 (Sigma-Aldrich, St Louis, MO) gradient. The mononuclear cell layer was washed with phosphate-buffered saline (PBS) solution, as instructed by the supplier. Aliquots of isolated PBMCs were slowly frozen to -80°C in freezing media consisting of 90% fetal bovine serum (FBS) and 10% sterile dimethyl sulfoxide (DMSO) prior analysis.

3.2 STATISTICAL ANALYSIS

SPSS software 17.0 (SPSS, Inc., Chicago, IL) was used for most of the statistical analyses. Stata software (Stata Corp, College Station, TX) was used for multivariate nonparametric quantile regression analyses in Paper I.

In Paper I, Shapiro-Wilk's test was used to evaluate whether the data was normal or non-normal distributed. Since most of the data were not normal distributed, non-parametric tests were used. The Mann-Whitney test was used for comparison between groups and for analysis of correlations of factors, Spearman's correlation test was used.

Shapiro-Wilk's test was also used in Paper II to evaluate the distribution of the data. Both parametric as well as non-parametric statistical methods were applied since both normal and non-normal distributed data were present. Within-groups comparisons (values before and after treatment) were performed by paired two-sampled t-test (normal distributed) or Wilcoxon signed rank test (non-normal distributions). Possible carry-over or period effects were investigated by comparing the sum and the differences of the responses between the sequence groups with unpaired two-sample student's t-test or Mann-Whitney. Any p-value of < 0.05 was considered as statistically significant. No adjustment for multiple testing was made. [65]

4 RESULTS

4.1 PAPER I; OBSERVATIONAL STUDY

The results from the observational study in CKD patients, both predialysis patients and dialysis patients are shown in Tables 3 and 4.

	Predialysis patients				Matched controls				Sign.
	Median	Mean	(min-max)	n	Median	Mean	(min-max)	n	
Age (years)	57	56	(33-66)	10	57	57	(36-69)	10	0.76
DNA damage									
DNA strand breaks (%DNA in tail)	8	8.9	(5.9-13.8)	10	6	6.4	(5.1-9.9)	9	*
Oxidative DNA lesions (%DNA in tail)	8	7.9	(2.4-14.4)	10	8.2	7.8	(6.1-10.1)	9	0.87
Salivary secretion									
Secretion rate at rest (mL/min)	0.2	0.3	(0.0-0.9)	10	0.3	0.3	(0.1-0.4)	9	0.653
Secretion rate, stimulated (mL/min)	1.6	1.9	(0.7-4.4)	10	1.8	1.8	(1.1-3.0)	9	0.683
Inflammation parameters									
IL-6 in saliva (pg/L)	15.5	16.1	(1.3-40.0)	9	5	14.5	(1.6-74.4)	8	0.63
hs-CRP in serum (mg/L)	5	10.8	(1.0-67.0)	10	1	1	(0.0-2.0)	10	**
Orosomuroid in plasma (g/L)	1.1	1.2	(0.8-2.0)	8	0.7	0.7	(0.6-0.9)	10	***
Haptoglobin in plasma (g/L)	1.4	1.8	(1.0-3.2)	8	0.8	0.7	(0.3-1.1)	10	**
LPC in blood (10 ⁹ /L)	8.5	9	(5.6-14.9)	10	6.1	7.5	(5.7-17.2)	10	0.075
Uremic state									
Urea in serum (mmol/L)	25.6	26.5	(12.9-36.6)	10	5.8	5.8	(4.9-6.8)	10	***
Creatinine in serum (μmol/L)	628	626	(341-937)	10	72	73	(54-95)	10	***
Albumin in serum (g/L)	38	37	(23-48)	10	40	40	(36-43)	10	0.062
Hemoglobin in blood (g/L)	121	119	(92-135)	10	148	148	(126-165)	10	***

Table 3. Predialysis patients and matched controls.

(* , ** and *** represent a p-value < 0.05, < 0.01 and < 0.001 respectively.)

	Dialysis patients				Matched controls				Sign.
	Median	Mean	(min-max)	n	Median	Mean	(min-max)	n	
Age (years)	63	62	(25-87)	69	62	63	(35-89)	69	0.821
DNA damage									
DNA strand breaks (%DNA in tail)	5.3	5.6	(3.4-13.2)	59	8.3	8.8	(4.7-18.4)	66	***
Oxidative DNA lesions (%DNA in tail)	7.6	8.4	(1.4-24.2)	59	8.2	8.6	(0.9-16.7)	66	0.515
Salivary secretion									
Secretion rate at rest (mL/min)	0.1	0.1	(0.0-0.5)	68	0.2	0.3	(0.0-1.4)	69	***
Secretion rate, stimulated (mL/min)	1.1	1.1	(0.0-2.5)	68	1.7	1.8	(0.3-5.2)	69	***
Inflammation parameters									
IL-6 in saliva (pg/L)	15	40.4	(0.2-154.5)	5	5.5	12.9	(0.2-91.5)	53	0.383
hs-CRP in serum (mg/L)	4.5	8.5	(1.0-77.0)	68	2	3.4	(1.0-33.0)	68	***
Orosomuroid in plasma (g/L)	0.9	1	(0.4-2.2)	67	0.8	0.8	(0.4-1.2)	68	***
Haptoglobin in plasma (g/L)	1.2	1.2	(0.1-2.6)	67	1.1	1.1	(0.2-2.4)	68	0.308
LPC in blood (10 ⁹ /L)	6.9	7.1	(1.8-14.6)	69	6.1	6.6	(4.2-14.0)	69	0.059
Uremic state									
Urea in serum (mmol/L)	20.5	21.7	(13.3-46.4)	69	5.8	6	(3.6-11.2)	67	***
Creatinine in serum (μmol/L)	716	730	(271-1333)	69	78	79	(48-118)	69	***
Albumin in serum (g/L)	35	35	(27-44)	68	39	39	(32-46)	69	***
Hemoglobin in blood (g/L)	125	123	(91-156)	69	146	146	(112-168)	67	***

Table 4. Dialysis patients and matched controls.

(*** represents a p-value < 0.001)

4.1.1 DNA damage

The level of DNA strand breaks in predialysis patients (Table 3) was significantly higher compared to matched controls ($p < 0.05$). Dialysis patients (Table 4) had significantly lower levels of DNA strand breaks compared to their matched controls ($p < 0.001$). There was also a significant difference between the levels in predialysis patients (higher) and the dialysis patients (lower) ($p < 0.001$). There were no significant differences between the groups in oxidative DNA lesions.

4.1.2 Salivary secretion rates

There were no significant differences in salivary secretion when comparing predialysis and their controls. The dialysis patients had significantly lower levels of saliva secretion both at rest ($p < 0.001$) and after stimulation ($p < 0.001$), compared to matched controls. The dialysis patients also had lower levels compared to the predialysis patients, both at rest ($p < 0.05$) and after stimulation ($p < 0.01$). There was a borderline correlation between salivary secretion at rest and DNA strand breaks in all CKD patients ($r_s = 0.224$, $p = 0.066$, $n = 68$). The correlation between salivary secretion at rest and after stimulation was significant in both CKD patients ($r_s = 0.600$, $p < 0.001$, $n = 78$) and in the controls ($r_s = 0.512$, $p < 0.001$, $n = 78$). In a multivariate quantile regression, the salivary secretion rate at rest predicted the level of DNA strand breaks significantly in CKD patients (estimate = 3.5, $p < 0.05$, $n = 68$). Age, sex, salivary secretion at rest, hs-CRP and creatinine were covariates in the model.

4.1.3 Inflammation parameters

All inflammation levels were higher in the CKD patients compared to the controls, however, not all were statistically significant. In the predialysis patients, hs-CRP ($p < 0.01$), orosomucoid ($p < 0.001$), haptoglobin ($p < 0.01$) were significantly higher in predialysis compared to matched controls. In the dialysis patients, hs-CRP ($p < 0.001$) and orosomucoid ($p < 0.001$) levels were higher compared to the matched controls. IL-6 in saliva was only measured in 14 CKD patients and there were no significant differences between the groups. The predialysis patients had significantly higher levels of LPC compared to dialysis patients ($p < 0.05$). Haptoglobin correlated significantly with oxidative DNA lesions in the dialysis patients ($r_s = 0.272$, $p < 0.05$, $n = 58$). In a multivariate quantile regression, hs-CRP predicted the level of DNA strand breaks

significantly in the controls (estimate = 0.18, $p < 0.05$, $n = 73$). Age, sex, salivary secretion at rest, hs-CRP and creatinine were covariates in the model.

4.1.4 Blood parameters

Creatinine ($p < 0.001$) and urea ($p < 0.001$) levels were higher in both predialysis and dialysis patients in comparison with the matched controls. Albumin concentrations were significantly lower ($p < 0.001$) in dialysis patients compared to controls. Hemoglobin levels were significantly lower in both dialysis patients and predialysis patients compared to their controls (both $p < 0.001$). Hemoglobin correlated negatively to DNA strand breaks in the control persons ($r_s = -0.294$, $p < 0.05$, $n = 73$).

4.1.5 Other findings

Oxidative DNA lesions were found to be sex-dependent; women (9.6 % DNA in tail) on dialysis had significantly higher levels ($p < 0.01$) compared to men (7.8 % DNA in tail). The same pattern was seen in the whole CKD group, women had significantly higher levels of oxidative DNA lesions when performing the multivariate quantile regression, with age, sex, salivary secretion at rest, hs-CRP and creatinine as covariates in the model (estimate = 2.2, $p < 0.05$, $n = 68$). Levels of DNA strand breaks were significantly higher ($p < 0.05$) in women (11.9 % DNA in tail) compared to men (7.6 % DNA in tail) in the predialysis patients.

4.2 PAPER II; INTERVENTION STUDY

The results from the intervention study (Paper II) on SBO supplementation in hemodialysis patients are shown in Table 5.

4.2.1 DNA damage

SBO treatment did not have a significant effect on the levels of DNA damage in the dialysis patients. The level of DNA strand breaks was 5.2 % DNA in tail (median value) both before supplement and placebo. The level of oxidative lesions did not change significantly, the baseline values were 7.7 % DNA in tail and 8.8 % DNA in tail before SBO supplement and placebo, respectively.

4.2.2 Salivary secretion rates

The baseline value of salivary secretion at rest was 0.05 mL/min before supplement and the stimulated production was 0.87 mL/min. The SBO treatment did not have any significant effect on these levels.

4.2.3 Inflammation parameters

SBO treatment did not have any significant effect on the inflammation markers hs-CRP, antitrypsin or orosomucoid in this study, baseline values 4.2 mg/L, 1.4 g/L and 1.0 g/L respectively. However there was a significant difference between the sequence groups, AB and BA ($p = 0.001$). The levels of hs-CRP increased 3.1 mg/mL in the AB group after SBO supplement, whereas the BA group decreased 0.50 mg/mL. The same pattern was seen in the orosomucoid levels ($p = 0.029$), the AB group increased 0.09 g/L whereas the BA group decreased 0.015 g/L.

4.2.4 Blood parameters

Creatinine and urea in plasma did not change significantly after SBO treatment. However, there was a significant increase in creatinine ($p = 0.030$) and urea ($p = 0.009$) levels after placebo treatment. The phosphate and sodium levels were significantly increased after SBO supplementation ($p = 0.02$ for both). Iron was significantly reduced after SBO supplementation ($p = 0.05$). CO₂-levels were not affected by SBO supplement, however, there was a significant decrease after placebo treatment ($p = 0.004$). The potassium level was, in opposite, increased after placebo treatment ($p = 0.03$). Also, the levels of immunoglobulin A and M were increased after placebo treatment ($p = 0.04$ resp. 0.01). The levels of albumin, glucose, thrombocytes, erythrocytes, leukocytes, haptoglobin, hemoglobin, iron saturation, transferrin, calcium, calcium-phosphate product and immunoglobulin G were not affected by SBO supplement or placebo. Carry-over effect was observed for iron levels, period effects were observed for CO₂, potassium, calcium-phosphate product and calcium levels.

	SBO supplement					
	Before			After		
	Mean	Median (min - max)		Mean	Median (min - max)	p
DNA damage						
DNA strand breaks (% DNA in tail)	5.5	5.2 (3.4 - 9.4)		5.3	5.1 (3.7 - 8.4)	0.23 ^a
Oxidative DNA damage (% DNA in tail)	9.1	7.7 (6.4 - 43.5)		8.9	8.8 (5.4 - 13.6)	0.12 ^a
Salivary secretion						
Secretion rate, at rest (ml/min)	0.1	0.1 (0.0 - 0.6)		0.1	0.0 (0.0 - 0.8)	0.29 ^a
Secretion rate, stimulated (ml/min)	1.0	0.9 (0.0 - 2.5)		1.0	0.9 (0.0 - 3.0)	0.46 ^b
Inflammation parameters						
C-reactive protein (hsCRP) (mg/l)	6.7	4.2 (0.2 - 65.0)		9.4	5.4 (0.4 - 77.2)	0.24 ^a
Antitrypsin (g/l)	1.5	1.4 (0.9 - 2.3)		1.5	1.5 (0.6 - 2.4)	0.48 ^b
Orosomucoid (g/l)	1.0	1.0 (0.6 - 2.1)		1.0	1.0 (0.6 - 1.8)	0.55 ^a
Blood parameters						
Albumin (g/L)	34.3	35.0 (21.0 - 44.0)		34.3	34.0 (25.0 - 44.0)	0.68 ^a
Calcium (Albumin corrected) (mmol/L)	2.4	2.5 (1.9 - 1.9)		2.4	2.4 (1.7 - 3.1)	0.43 ^b
Calcium x phosphate product	3.7	3.5 (2.1 - 6.4)		3.9	3.6 (2.1 - 6.5)	0.15 ^b
Carbon dioxide (mmol/L)	24.3	24.0 (15.0 - 32.0)		23.9	24.0 (19.0 - 30.0)	0.39 ^a
Creatinine (micromol/L)	746	690 (345 - 1293)		761	765 (384 - 1308)	0.39 ^b
Erythrocytes (10 ¹² /L)	3.8	3.8 (2.7 - 4.7)		3.8	3.8 (2.8 - 4.8)	0.96 ^b
Glucose (mmol/L)	5.9	5.4 (4.1 - 11.0)		5.9	5.4 (2.2 - 11.0)	0.99 ^a
Haptoglobin (g/L)	1.1	1.0 (0.1 - 2.9)		1.2	1.1 (0.1 - 2.5)	0.30 ^b
Hemoglobin (g/L)	120	120 (95 - 156)		120	122 (89 - 145)	0.82 ^b
Immunoglobulin A (g/L)	2.6	2.5 (0.1 - 6.4)		2.7	2.4 (0.1 - 6.3)	0.46 ^a
Immunoglobulin G (g/L)	11.3	11.0 (4.8 - 21.9)		11.6	10.8 (5.5 - 24.1)	0.13 ^a
Immunoglobulin M (g/L)	0.7	0.7 (0.1 - 3.5)		0.8	0.7 (0.2 - 3.8)	0.21 ^a
Iron (micromol/L)	12.0	11.0 (4.0 - 32.0)		10.7	10.0 (5.0 - 25.0)	* ^a
Iron saturation	0.3	0.2 (0.1 - 0.7)		0.2	0.2 (0.1 - 0.6)	0.05 ^a
Leukocytes (10 ⁹ /L)	7.2	6.8 (1.8 - 14.6)		7.1	6.9 (2.8 - 11.2)	0.93 ^a
Phosphate (mmol/L)	1.5	1.5 (0.8 - 2.5)		1.7	1.7 (0.9 - 2.7)	* ^b
Potassium (mmol/L)	5.1	5.2 (2.8 - 6.9)		5.0	4.9 (3.2 - 9.6)	0.36 ^a
Sodium (mmol/L)	138	137 (132 - 146)		139	138 (132 - 146)	* ^a
Thrombocytes (10 ⁹ /L)	218	214 (54 - 374)		221	212 (70 - 389)	0.61 ^b
Transferrin (g/L)	1.8	1.8 (0.8 - 2.7)		1.8	1.8 (1.0 - 2.6)	0.87 ^b
Urea (mmol/L)	22.2	21.2 (6.0 - 47.8)		22.9	22.8 (8.9 - 39.7)	0.40 ^a

	Placebo treatment					
	Before			After		
	Mean	Median (min - max)		Mean	Median (min - max)	p
DNA damage						
DNA strand breaks (% DNA in tail)	5.5	5.2 (3.6 - 13.2)		5.9	5.3 (3.6 - 11.5)	0.20 ^a
Oxidative DNA damage (% DNA in tail)	9.0	8.8 (4.6 - 24.2)		8.6	8.2 (4.1 - 13.7)	0.89 ^b
Salivary secretion						
Secretion rate, at rest (mL/min)	0.1	0.1 (0.0 - 0.5)		0.1	0.1 (0.0 - 0.9)	0.23 ^a
Secretion rate, stimulated (mL/min)	1.1	1.1 (0.0 - 2.4)		1.1	0.9 (0.0 - 3.3)	0.97 ^a
Inflammation parameters						
C-reactive protein (hsCRP) (mg/L)	10.1	5.0 (0.4 - 77.0)		9.0	4.5 (0.2 - 95.9)	0.63 ^a
Antitrypsin (g/L)	1.5	1.6 (0.8 - 2.1)		1.5	1.5 (0.7 - 2.1)	0.37 ^b
Orosomucoid (g/L)	1.1	1.0 (0.4 - 2.0)		1.0	1.0 (0.6 - 1.6)	0.31 ^a
Blood parameters						
Albumin (g/L)	34.4	34.0 (22.0 - 43.0)		34.6	35.0 (25.0 - 41.0)	0.60 ^b
Calcium (Albumin corrected) (mmol/L)	2.4	2.5 (2.1 - 2.9)		2.5	2.5 (2.2 - 2.7)	0.43 ^b
Calcium x phosphate product	4.0	3.9 (1.7 - 6.9)		4.2	3.9 (2.1 - 7.5)	0.33 ^b
Carbon dioxide (mmol/L)	24.4	24.0 (20.0 - 29.0)		23.1	24.0 (14.0 - 29.0)	** ^a
Creatinine (micromol/L)	719	693 (346 - 1078)		774	751 (400 - 1402)	* ^b
Erythrocytes (10 ¹² /L)	3.8	4.0 (2.9 - 5.0)		3.9	3.8 (2.8 - 4.9)	0.18 ^a
Glucose (mmol/L)	6.0	5.4 (4.2 - 11.0)		6.3	5.2 (3.9 - 20.2)	0.40 ^a
Haptoglobin (g/L)	1.3	1.2 (0.1 - 2.7)		1.2	1.2 (0.1 - 2.7)	0.61 ^b
Hemoglobin (g/L)	120	121 (94 - 149)		123	123 (91 - 159)	0.27 ^b
Immunoglobulin A (g/L)	2.7	2.6 (0.1 - 5.6)		2.8	2.5 (0.1 - 6.3)	* ^b
Immunoglobulin G (g/L)	11.2	10.7 (4.9 - 22.0)		11.4	10.9 (5.4 - 5.4)	0.33 ^b
Immunoglobulin M (g/L)	0.7	0.7 (0.1 - 3.2)		0.8	0.7 (0.1 - 3.6)	** ^a
Iron (micromol/L)	12.2	11.0 (5.0 - 38.0)		11.4	10.0 (7.0 - 22.0)	0.74 ^a
Iron saturation	0.3	0.2 (0.1 - 0.7)		0.3	0.2 (0.1 - 0.6)	0.73 ^a
Leukocytes (10 ⁹ /L)	7.1	7.1 (2.6 - 11.0)		7.1	6.8 (3.3 - 12.6)	0.82 ^b
Phosphate (mmol/L)	1.7	1.6 (0.9 - 3.2)		1.8	1.8 (0.9 - 3.1)	0.46 ^a
Potassium (mmol/L)	5.0	4.9 (3.1 - 6.6)		5.4	5.4 (3.7 - 8.4)	* ^b
Sodium (mmol/L)	138	138 (131 - 150)		138	138 (128 - 143)	0.54 ^b
Thrombocytes (10 ⁹ /L)	213	189 (59 - 343)		211	204 (106 - 395)	0.81 ^a
Transferrin (g/L)	1.8	1.8 (1.1 - 2.6)		1.8	1.8 (1.1 - 2.9)	0.15 ^b
Urea (mmol/L)	21.8	22.0 (8.2 - 40.0)		24.5	24.6 (10.9 - 54.3)	** ^a

a: Non-normal distribution
b: Normal distribution

Table 5. Results from the intervention study. Levels of DNA damage, salivary secretion, inflammation and blood markers in hemodialysis patients before and after sea buckthorn oil supplementation and placebo. (* and ** represent p-value <0.05 and <0.01 respectively.)

5 DISCUSSION

It is well established that CKD patients have elevated levels of inflammation and oxidative stress. This is due to failure of the kidney to excrete waste products, imbalance in the regulation of salt and water in the body, antioxidative deficiency and malnutrition. In dialysis patients, the dialysis treatment itself also causes increased inflammation and oxidative stress levels. Oral complaints including inflammation in the oral cavity and changes in saliva production and constitution have been reported in CKD patients [49, 66]. These alterations can contribute to systemic inflammation and the increased risk for cardiovascular disease.

In the observational study (Paper I) we investigated inflammation parameters mainly in blood, but also in saliva. The results are consistent with earlier studies regarding inflammation. All parameters showed higher values on systemic inflammation in CKD patients compared to matched controls. The level of inflammation in saliva, assessed by the concentration of IL-6, was also higher in CKD patients but it was only measured in few patients and the difference was not statistically significant. Only one significant difference in inflammation between dialysis patients and predialysis patients was found, predialysis patients showed higher levels of LPC compared to dialysis patients.

One aim with the study was to investigate oral health in CKD patients and we wanted to assess the oxidative stress levels in the minor accessory salivary glands by measuring oxidative DNA damage. Damage to the salivary glands can cause decreased saliva production and symptoms of dry mouth. In patients with Sjögren's syndrome (an autoimmune disease leading to the destruction of salivary and lacrimal glands), Ryo *et al* showed increased levels of 8-OHdG in the saliva, suggesting a role of oxidative stress in the salivary gland destruction [67]. Earlier studies on CKD patients have shown high levels of DNA damage in PBMCs [32, 68], however DNA damage in peripheral tissue in CKD patients has not been extensively studied and this study was, to our knowledge, the first study that measured DNA damage in the salivary glands. In Paper I, it was found that predialysis patients had significant higher levels of DNA damage compared to matched controls. This is in accordance with the earlier studies showing elevated levels of DNA damage in PBMCs. On the contrary, the findings in dialysis patients were the opposite; they had lower DNA damage compared to both the matched controls and the predialysis patients. Explanations to the lower levels of DNA damage in dialysis patients compared to the predialysis patients could be that the

dialysis treatment, with purification of the blood enabling removal of toxic compounds that can induce DNA damage. It has been shown that the dialysis treatment itself can cause ROS formation by activation of neutrophils due to both bioincompatible dialyzer and contaminated dialysate. However, since the samples in our study were collected just before the dialysis session, acute effects of neutrophil activation were not likely to have a major influence on the results.

The lower levels of DNA damage in dialysis patients compared to their matched control are more difficult to explain. It is possible that the systemic inflammation and oxidative stress enhance the DNA repair and/or antioxidative enzymes. Herman *et al* showed increased levels of spontaneous DNA repair just after hemodialysis treatment, in response to increased DNA damage [69]. While the inflammation and oxidative stress in the blood system is enhanced, the level in peripheral tissue might be differently affected. Bibi *et al* found higher levels of the antioxidant enzymes SOD and peroxidase in saliva from peritoneal dialysis patients compared to predialysis patients [70]. In contrast, SOD-activity in erythrocytes was shown to be decreased in dialysis patients compared to controls [71]. Considering these observations, it is possible that in dialysis patients, the enhanced systemic inflammation and oxidative stress stimulate antioxidative processes differently in peripheral tissue compared to circulating blood cells.

Dialysis patients had lower levels of unstimulated and stimulated salivary flow rates compared to both controls and predialysis patients. Earlier studies have shown similar results, Gavalda *et al* showed a significant decrease in stimulated saliva among dialysis patients compared to controls and Kho *et al* showed decreased levels of saliva in rest and stimulated parotid saliva [48, 72]. Damage to the salivary gland and/or restricted fluid intake were proposed underlying mechanisms. Hyposalivation could be an explanation to the higher prevalence of oral complaints since the saliva is important for lubrication, bacterial defence, buffering capacity, taste and digestion.

In the intervention study we applied a crossover study design and it has the advantage of acquiring fewer study subjects since the patients are their own controls. It also decreases the influence of patient variation and covariates. The crossover design is based on the assumptions that the disease condition is stable during the study and that the study period is short enough to avoid period effects. In addition, the effect of the intervention should not be permanent and a wash-out period between the treatment periods should be long enough to prevent carry-over effects. Violence to the assumptions might result in incorrect data analysis. In our intervention study, the

kidney disease was assumed to remain stable during the study period of in total 20 weeks. However, we detected period effects of some parameters, including CO₂, potassium, calcium-phosphate product and calcium levels. Further, despite a wash-out period of four weeks, we also detected a carry-over effect on iron levels. In our statistical analysis we did not correct for multiple comparison, if adjustments on p-values had been carried out, a majority of the period effects had not been statistically significant. Crossover studies are also more sensitive to drop-outs compared to parallel study designs since paired analysis is performed. In the study we had 16 drop-outs, due to deaths or patients that were not interested in additional biopsies taken, which limited the study.

Studies have reported health effects from intake of sea buckthorn in terms of decreased levels of inflammation markers and ameliorated effects on dry eyes symptoms. Antioxidative effects have been shown in *in vitro* studies and animal models [57, 58, 73]. Based on these findings, the aim with the intervention study was to investigate if supplementation with SBO could have impact on DNA damage in minor accessory glands, inflammation or salivary flow rates in CKD patients. The results from this study did not show any effects on the levels of DNA damage after SBO supplementation. An explanation to the absence of effect could be that the levels of DNA damage in the hemodialysis patients actually were lower than expected, as found in Paper I. Earlier results from studies on antioxidant supplementation indicate that normal levels of oxidative stress might not be affected by antioxidant supplementation, whereas certain risk groups with elevated levels of oxidative stress might be. As previous studies on CKD patients have shown increased levels of DNA damage in PBMCs, it could be speculated that enhanced levels of DNA damage in circulating blood cells might have been beneficially affected by supplementation with SBO. Further, the lack of antioxidative effect in this clinical study points out the limitations in extrapolating *in vitro* findings on antioxidative mechanisms to *in vivo* systems.

Even though the salivary flow rates were lower in the dialysis patients compared to in the controls, discussed in Paper I, SBO did not improve the saliva production in the clinical study as hypothesised. While Larmo *et al* showed an effect of sea buckthorn on CRP levels (median reduction was -0.059 mg/L, p = 0.039) we did not detect any significant change in the inflammation markers after SBO supplementation [61]. However, the reduction of the CRP level in the study of Larmo *et al* was small and the conflicting results might also be due to the difference in supplement material, in their study sea buckthorn puree was administered, with a higher

content of water-soluble compounds. In another study of Larmo *et al*, where they administered SBO, they could detect an attenuation of increased tear film osmolarity [62]. While they could not detect any changes in the fatty acid content of the tear film, they proposed a mechanism involving modulation of the local inflammation status of dry eyes. However, they did not detect any changes on systemic biomarkers of inflammation. These findings further point out the contradictions in nutritional supplements versus dietary intake of fruits and vegetables, possibly explained by the importance of synergy between vitamins, antioxidants and phytochemicals for positive health effects.

In contrast to the inflammation markers, some blood markers were actually affected by SBO supplementation; iron levels were reduced, sodium and phosphate levels were increased. A reduction of iron levels is a negative health effect since iron is important for oxygen transport and iron deficiency might lead to anaemia. Sodium is an important osmolyte and alterations in serum levels of sodium have impact on osmosis and cellular functions. Increased phosphate levels are also potentially harmful for CKD patients. Hyperphosphatemia stimulates the vascular calcification, a risk factor for developing atherosclerosis and cardiovascular disease [74]. However, the changes were small and might not be of clinical relevance. In addition, the placebo treatment had impact on some parameters; creatinine, urea, potassium, IgA and IgM increased and CO₂ decreased after administration of coconut placebo oil. Coconut oil is a common placebo treatment used when studying effects of fatty acid supplementation. However, since creatinine and urea accumulate in blood when the kidney function is impaired, these findings suggest that a different placebo substance should be used in future clinical studies on CKD patients.

6 CONCLUSION

The results from the studies in this thesis showed:

- Increased systemic inflammation in CKD patients compared to controls.
- Higher levels of creatinine and urea in CKD patients compared to controls.
- Lower levels of DNA strand breaks in the minor accessory salivary glands in hemodialysis patients compared to both controls and predialysis patients.
- Higher levels of DNA strand breaks in the minor accessory salivary glands in predialysis patients compared to controls.
- Lower saliva production in CKD patients compared to controls.
- Positive correlation between oxidative DNA lesions and haptoglobin levels in dialysis patients.
- No significant effect of sea buckthorn oil supplementation on DNA damage, inflammation or saliva production in hemodialysis patients.
- The plasma levels of phosphate and sodium increased and iron levels decreased after sea buckthorn oil supplementation.

In conclusion; oxidative stress and inflammation are important risk factors that contribute to disease progression and mortality in chronic kidney disease patients. The interrelations between these events are complex and factors including dialysis treatment, medication, diet and oral health are of importance. In our study we found that despite elevated systemic inflammation, the levels of DNA damage in minor accessory salivary glands in dialysis patients were lower compared to controls. The results suggest the involvement of DNA repair and antioxidative mechanisms in this tissue. Supplementation with sea buckthorn oil did not show any reduction on DNA damage or inflammation in dialysis patients, concluding that sea buckthorn oil supplementation did not have any beneficial health effects in our study group.

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